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ORIGINAL ARTICLE

Detection of c-kit mutational status in small-cell lung cancer in a Chinese cohort

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Abstract

Background: Overexpression of KIT (CD117), a tyrosine kinase receptor, and its natural ligand, stem cell factor, are found in small-cell lung cancer (SCLC). Somatic mutations of the proto-oncogene c-kit constitutively activate KIT expression in a ligand-independent way. To explore the clinical value of the c-kit mutation as a potential target for therapy with tyrosine kinase inhibitors, the c-kit mutational status and KIT expression in tumors from Chinese patients with SCLC were analyzed.

Methods: Using 107 paraffin-embedded SCLC tumor specimens, c-kit exons 9, 11, 13, and 17 were analyzed for mutations by polymerase chain reaction and direct sequencing.

Results: There were no activating mutations in exons 9, 11, 13, or 17. However, a point mutation in intron 16 (81240 G>A) was found in 11 out of the 107 samples (10.3%), of which the majority were limited-stage SCLC (10/11, 90.9%). Immunohistochemical staining of tumors harboring the c-kit point mutation using the anti-CD117 antibody showed that the mutation status was not associated with the expression of KIT.

Conclusion: These findings indicate that the incidence and the types of c-kit mutations in SCLC tumors found in Chinese are different from those of the Caucasian population. Nevertheless, c-kit mutations are similarly rare in both groups, implying that they may not be suitable targets for c-kit-based tyrosine kinase inhibitors.

Introduction

In China, it is estimated that about 100 000 new cases of small-cell lung cancer (SCLC) occur annually.[1] SCLC has unique biological characteristics, including rapid tumor doubling time, high growth rate, and early development of widespread metastases. Although SCLC is highly sensitive to initial chemotherapy and radiotherapy, most patients finally die from disease relapse with a five-year survival rate of only five percent.[2] Within the last 30 years, therapeutic strategies for SCLC treatment have been explored, however, the prognosis for SCLC patients has not been substantially improved. Thus, a better understanding of the molecular mechanisms involved in the initiation, development, and metastasis of SCLC is needed to improve the outcomes of SCLC treatment.

A variety of receptor tyrosine kinases and growth factors have been implicated in both pathogenesis and prognosis of SCLC, including c-met, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and KIT.[3] Upon binding of its ligand stem cell factor (SCF), KIT protein (CD117) is activated through dimerization resulting in the phosphorylation of JAK–STAT, PI3K and MAPK, thus, triggering intracellular signal transduction. Studies have shown that KIT protein expression and kinase activity contribute to a number of processes related to the growth and progression of many malignancies, including SCLC. Autocrine or paracrine activation of the KIT receptor by SCF has been postulated for lung cancer. The receptor can also be constitutively activated in a ligand-independent way through specific mutation of the c-kit gene. KIT protein expression was found in 79–88% of SCLC cell lines and the tyrosine kinase inhibitor imatinib had an inhibitory effect on SCLC cell growth by the inactivation of KIT.[4–6] Nevertheless, there was no observed antitumor activity in several phase II clinical trials conducted

Table 1 Primer sequences for detecting exon 9, 11, 13, and 17 of c-kit oncogene

	Forward sequence	Reverse sequence
Exon 9	ACTGTAAAACGACGGCCAGTTAAAAGTATGCCACATCCCAAG	ACCAGGAAACAGCTATGACCCATGGTCAATGTTGGAATGAAC
Exon 11	ACTGTAAAACGACGGCCAGTTCCAGAGTGCTCTAATGAC	ACCAGGAAACAGCTATGACCAGGTGGAACAAAACAAAGG
Exon 13	ACTGTAAAACGACGGCCAGTAAGATGCTCAAGCGTAAGTTC	ACCAGGAAACAGCTATGACCAAGCAGTTTATAATCTAGCATTGCC
Exon 17	ACTGTAAAACGACGGCCAGTGTGAACATCATTCAAGGCGT	ACCAGGAAACAGCTATGACCCCTTTGCAGGACTGTCAAGCA

in SCLC patients, leaving inconclusive results of the effect of imatinib against SCLC with the targeted KIT receptor.[7,8] Several studies have illustrated the mutational status of the c-kit gene in Caucasian cohorts with small case numbers or limited domains,[4,9,10] but no determination of the c-kit mutational status has yet been reported in Chinese patients with SCLC. To identify the frequency of c-kit mutations in Chinese SCLC patients and further clarify whether or not KIT is a candidate for molecular-targeted therapy, we examined SCLC tumors from Chinese patients in terms of c-kit mutations in a variety of domains, determined KIT expression levels, and correlated c-kit mutation/expression with clinical characteristics.

Materials and methods

Patients and tumor samples

Qualified specimens with SCLC treated at Jilin Province Cancer Hospital from 2006 to 2009 were analyzed in this study. All patients had histologically or cytologically diagnosed SCLC. Formalin-fixed and paraffin-embedded (FFPE) tumor samples were retrieved from the Department of Pathology. The clinical data for all patients were collected from their medical records. The Ethical Review Board of our hospital approved this study. Of the tumor specimens used, 99 were from primary tumors removed by surgery or bronchoscopy and eight were from metastases of confirmed primary SCLC patients. Furthermore, FFPE gastrointestinal stromal tumor (GIST) specimens were used as positive control for c-kit mutation.

Mutational analysis of c-kit

Mutations in the juxtamembrane domains (exons 9 and 11) and tyrosine kinase domains (exons 13 and 17) were analyzed in this study by polymerase chain reaction (PCR) and direct DNA sequencing. Tumor genomic DNA was isolated from a total of 107 tumors. A series of sections (5 μm thick) were made from each block, and one slide was used for hematoxylin and eosin staining to ensure a tumor-cell-rich area. Genomic DNA was extracted from the additional five slides using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hamburg, Germany) according to the manufacturer's instructions. Primers for exons 9, 11, 13, and 17 amplifications were designed, as previously reported, with a minor modification (Table 1), and synthesized at Sangon Biotech Co. Ltd (Shanghai, China). The settings for gene amplification were 94°C for four minutes followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds, with an extra extension step at 72°C for 10 minutes after the last cycle. Direct sequencing was performed at Sangon Biotech Co. Ltd. All mutations were confirmed by both forward- and reverse-direction sequencing.

Immunohistochemical staining and quantification

The slides were deparaffinized in xylene and rehydrated through graded alcohols to distilled water. Antigen retrieval was performed by heating tissue sections in ethylenediamine-tetraacetic acid (EDTA) buffer (1 mM, pH 9.0) in a pressure cooker for one minute. Endogenous peroxidase was blocked with hydrogen peroxide (3%) for 10 minutes and Tris-buffered saline plus Tween 20 (0.05%, pH 7.4) was used for all washes and diluents. Slides were blocked with universal blocking serum (Santa Cruz, Beijing, China) for 60 minutes, and after being washed, the primary antibody (anti-CD117, Santa Cruz, Beijing, China) was added to the slides and incubated at 4°C overnight, followed by the second antibody. The slides were briefly counterstained with hematoxylin. GIST samples were used as controls in the experiments. The CD117 positivity was evaluated considering both percentage of positive cells and staining intensity. Negative (−) represents no stained cells in the samples; weak positive (+) represents ≤10% of cells with weak staining intensity; moderate positive (++) represents >10 and ≤50% of cells with weak to moderate staining intensity; strong positive represents >50% of cells with moderate to strong staining.

Statistical analysis

Statisticians performed all statistical analyses using SPSS 12.1 software. The chi-square test was used for statistical analysis of categorized data. The Kaplan-Meier method was used to estimate the distribution of survival. The log-rank test was used to compare the distribution between different groups. The Cox proportional-hazards model was used to estimate

Table 2 Clinical characteristics of patients with small-cell lung cancer (SCLC)

	Enrolled patients	
	Number	%
Age	57.62 ± 8.77	
Gender		
Male	74	69.2
Female	33	30.8
Stage at diagnosis		
Limited	80	74.8
Extensive	27	25.2
Smoking status		
Never smoker	48	45
Light smoker	14	13.1
Smoker	45	42.1
Performance status		
0–1	85	79.4
2–3	22	20.6
Surgery		
Yes	33	30.8
No	74	69.2
Chemotherapy		
Etoposide + Cisplatin	77	72
Etoposide + Carboplatin	3	2.8
Other	27	25.2
Radiotherapy		
Yes	28	26.2
No	79	73.8
Treatment efficacy		
Complete response	28	26.2
Partial response	33	30.8
Stable disease	22	20.6
Progressive disease	24	22.4
Survival		
1-year	76	71
2-year	31	29
Overall survival (months)	13.19 ± 0.92	

Table 3 Univariate analyses for survival of patients with small-cell lung cancer (SCLC)

	Univariate analyses		
	RR	95% CI	*P*-value
Age			
<65	1		
≥65	0.901	0.492~1.653	0.737
Gender			
Female	1		
Male	1.024	0.619~1.693	0.926
Smoking status			
Never smoker	1		
Light smoker	1.325	0.603~2.914	0.484
Smoker	1.107	0.667~1.839	0.694
Performance status			
0–1	1		
2–3	1.293	0.728~2.928	0.042
Surgery			
No	1		
Yes	0.467	0.259~0.844	0.007
Chemotherapy			
Etoposide + Cisplatin	1		
Etoposide + Carboplatin	0.845	0.203~3.506	0.816
Other	2.512	1.384~4.559	0.002
Radiotherapy			
No	1		
Yes	0.436	0.236~0.805	0.038
Treatment efficacy			
Complete + partial response	1		
Stable disease	1.555	0.708~3.414	0.271
Progressive disease	1.928	0.960~3.872	0.045
Stage at diagnosis			
Limited	1		
Extensive	1.369	0.810~2.313	0.009
Metastasis			
No	1		
Yes	1.214	0.748~1.970	0.433

CI, confidence interval; RR, response rate.

hazard ratios and 95% confidence intervals (CIs), as well as the effect of prognostic factors on survival. *P* values of <0.05 were considered statistically significant.

Results

Clinicopathological characteristics and survival

The clinicopathological parameters of all patients in this study are listed in Table 2. A total of 150 cases were screened, of which 109 cases had adequate tumor tissues for gene amplification and 107 cases had complete medical records. Patients were randomly selected from inpatients treated at Jilin Province Cancer Hospital with the ratio of males to females at 2:1. The skewed ratio was likely a result of different smoking habits between genders, given that majority of smokers are male in China and it is well established that SCLC is in part attributable to cigarette smoking.[11] According to the Veteran's Administration Lung Group 2-stage classification scheme, the clinical stage was limited-stage in 80 patients and extensive-stage in 27 patients. Most of the patients (89.7%) received at least one therapeutic treatment, such as surgery, platinum-based chemotherapy or radiotherapy. The one and two-year survival rates of all patients were 71% and 29%, respectively, and the overall survival (OS) time was 15 months (95% CI, 12.7–19.3 months). Univariate analysis showed that disease stage ($P = 0.009$), performance status of the patients ($P = 0.042$), and systemic treatment ($P < 0.05$) were associated with survival (Table 3). One and two-year survival rates of patients who were limited-stage versus

Table 4 The c-kit mutation and KIT expression in small-cell lung cancer (SCLC)

	c-kit mutation			KIT expression		
	Positive	Negative	*P* value	Low (0–1)	High (2–3)	*P* value
Gender						
Male	7	67		33	41	
Female	4	29	0.721	14	19	1.000
Age						
<65	10	74		37	47	
≥65	1	22	0.684	10	13	1.000
Smoking						
Never smoker	5	43		21	27	
Light smoker	1	13	0.785	6	8	0.080
Smoker	5	40	0.326	20	25	0.163
Performance status						
0–1	10	75		38	47	
2–3	1	21	0.445	9	13	1.000
Disease stage						
Limited-stage	10	70		34	46	
Extensive-stage	1	26	0.429	13	14	0.637
Metastasis						
Yes	6	55		27	34	
No	5	41	1.000	20	26	0.478
Progression-free survival (months)						
<3	1	8		4	5	
3–6	3	23	0.227	12	14	1.000
>6	7	65	0.186	31	41	0.421
Overall survival (months)						
<12	4	44		21	27	
12–24	5	38	0.592	19	24	0.806
>24	2	14	0.264	7	9	1

extensive-stage were 73.6% and 38.5% versus 51.2% and 9.3%, respectively.

C-kit mutation and clinicopathological parameters

In order to investigate the unique mutational situation of SCLC in a Chinese population, genomic DNA was extracted from the tumor samples and amplified by PCR, followed by direct sequencing. The clinicopathological characteristics of the patients with c-kit mutations are shown in Table 4. No missense mutations were found in exons 9, 11, 13, or 17; however, a point mutation in intron 16 (81240 G > A) was found when the c-kit gene was amplified using specific primers targeting exon 17 (Fig. 1). Among all tumor specimens, 11 samples (11/107, 10.3%) contained the point mutation, of which 10 samples were limited-stage SCLC (Fig. 1 and Table 4). The mutation incidences in limited-stage versus extensive-stage were 12.5% versus 3.7%, respectively. Interestingly, mutation incidence was significantly higher in patients over age 65 compared with those under 65 (11.9% vs. 4.3%). Further analyses showed that the c-kit mutation rate was not affected by the gender, smoking status, or performance status of patients (Table 4). Multivariate analysis demonstrated that c-kit mutation was not associated with survival ($P > 0.05$). The median progression-free survival was nine months (range, 3–40 months) in the c-kit mutation-positive group and seven months in the mutation-negative group

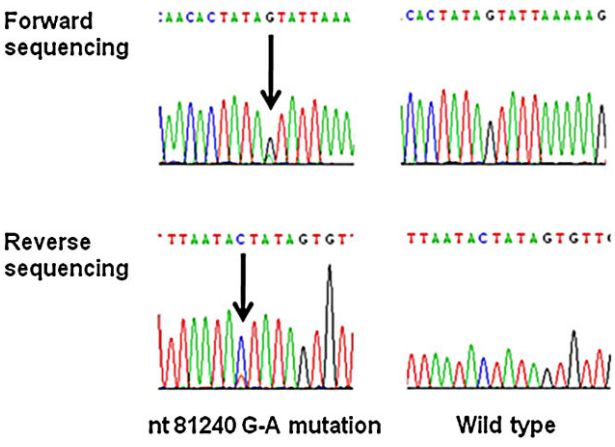

Figure 1 Representative electropherogram of heterozygous mutation of c-kit intron 16 in small-cell lung cancer (SCLC).

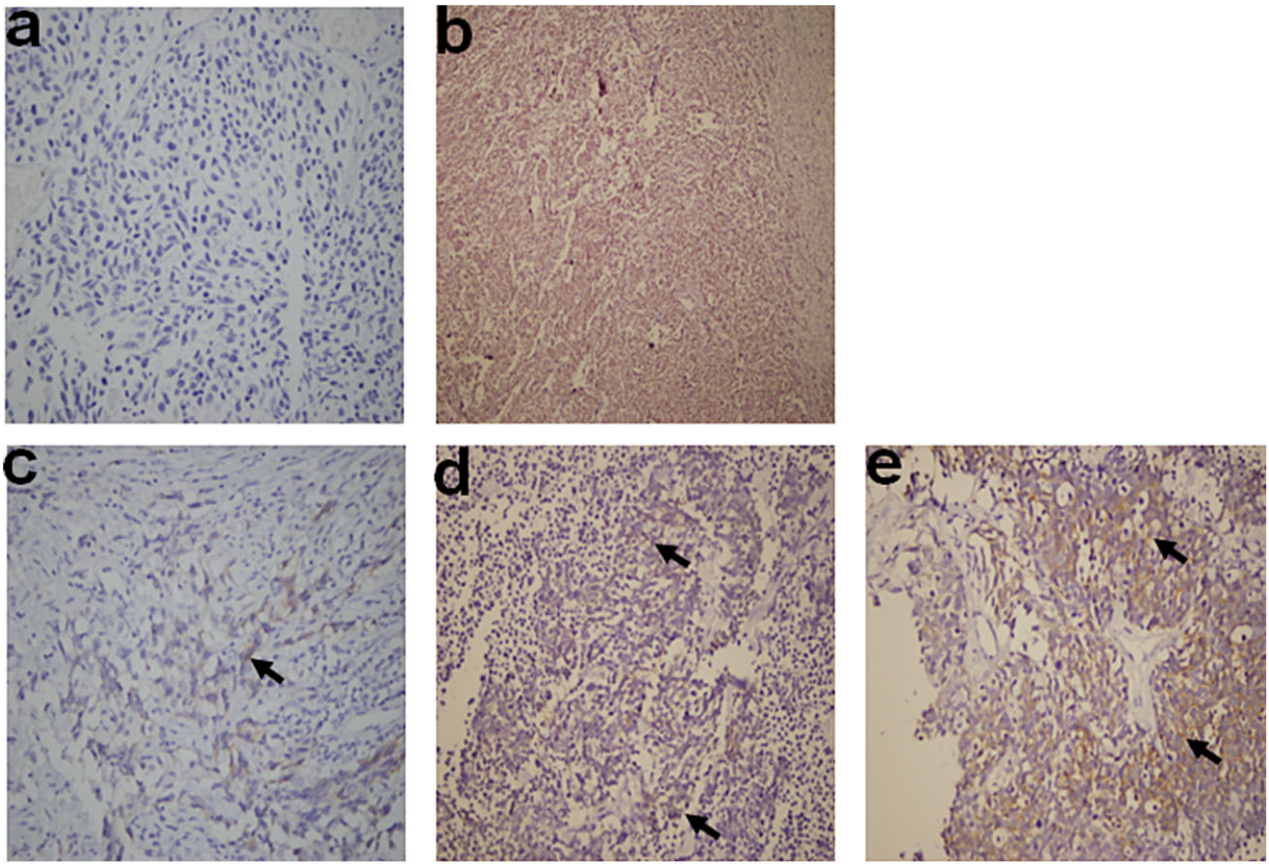

Figure 2 Representative pictures for CD117 immunohistochemical staining. Dark color and arrows indicat positive staining. (a) CD117-negative sample; (b) Representative CD117-positive gastrointestinal stromal tumor sample; (c) Weak CD117-positive SCLC sample (+); (d) Moderate CD117-positive SCLC sample (++); (e) Strong CD117-positive small-cell lung cancer (SCLC) sample (+++). Original magnification, 400×.

(range, 4–35 months), whereas the OS was similar between mutation-positive and mutation-negative groups (14.5 months vs. 14 months, $P > 0.05$), suggesting that mutation status is not a determining factor for OS.

KIT expression and clinicopathological parameters

To further explore the functional consequences of the intron mutation, KIT expression by immunohistochemistry using specific antibodies was conducted (Fig. 2). Of 11 specimens harboring the mutation, staining density was weak (score 1) in two cases, moderate (score 2) in two cases and strong (score 3) in four cases, whereas three cases did not show KIT expression. Of all tumor samples, KIT expression with moderate to strong density versus no staining (negative) was 56.1% (60/107) versus 22% (24/107). Statistical analyses revealed that there was no correlation between KIT expression and the intron mutation (Table 4).

Discussion

Thriving discovery with respect to tyrosine kinase receptors in other cancers inspired basic and clinical research on similar signaling pathways in SCLC. The KIT tyrosine kinase activity can be constitutively activated in a ligand-independent manner through specific mutations in the c-kit oncogene. Furthermore, genetic mutations were successful predictors for TKI treatment, notably EGFR mutations for patients with non-SCLC (NSCLC) and c-kit mutations for patients with GIST.[12,13] To the best of our knowledge, three studies of c-kit genomic changes in SCLC showed that mutations of the c-kit gene exist in Japanese and Caucasian cohorts, however those studies provided limited data. Sekido *et al*. examined 15 SCLC cell lines, as well as 13 SCLC primary tumor samples, and reported that there was a single c-kit mutation in codon 541 and that this *nonsynonymous* mutation occurred within the transmembrane domain with a mutation rate of only 6.7% (1/15) and 7.7% (1/13) of SCLC cell lines and primary

tumor specimens, respectively.[10] Burger *et al*. then examined the mutations of c-kit restricted to exon 11 in 26 SCLC cases and identified no mutations in KIT positive SCLC tumor samples.[9] At the same time, Boldrini *et al*. tested 60 SCLC samples and explored two mutations in exon 9 and three mutations in exon 11.[4] DNA aberrations in exon 13 or in exon 17 have not been reported previously.

It is well established that ethnic differences affect the incidence of various cancers. For NSCLC, EGFR mutations are found more frequently in East Asians, while the KRAS mutation rate is lower in East Asians than Caucasians. The highest frequency of MET mutations was also found in East Asians.[14,15] In light of these observations, we hypothesized that a Chinese population might present different mutations of the c-kit oncogene in terms of incidence, mutational sites or functional consequence. In our present work, we expanded the mutational analysis of the c-kit oncogene to exons 9, 11, 13, and 17 in Chinese patients with SCLC. Unfortunately, we failed to detect any mutations in exons 9 or 11, a distinct difference from what has been found in Caucasians.[9] We speculate that the reasons lie in the ethnic difference, rather than experimental error, because we employed GIST tumor samples as controls throughout the entire study to provide confidence in the PCR amplification and sequencing techniques. For exons 13 and 17, we did not observe any activating mutations. However, we identified a point mutation within intron 16 when we performed DNA sequencing targeting exon 17. To our knowledge, this is the first analysis of c-kit mutation beyond exons 9 and 11. As this mutation has not been described in neoplasms of the lungs, we further explored the functionality of the mutant gene, that is, the correlation of the mutation with KIT expression using anti-CD117 antibody by immunohistochemistry in tumor tissues. The results showed a wide distribution level of KIT protein expression in the tumor specimens bearing the c-kit mutation, implying the mutation discovered in intron 16 was not likely contributing to the overexpression of KIT, as was concluded with other c-kit mutations. Given the low incidence of the mutation (11/110) and the lack of functional effect on KIT expression, the real consequences of the intron 16 mutation described in this study need to be further and accurately clarified.

To further explore the clinical value of the intron mutation, we examined the correlation of the mutation to clinical characteristics of the patients enrolled in the study. Interestingly the results revealed that the point mutation within intron 16 of c-kit gene correlated with disease stage, with 12.5% (10/80) incidence in limited stage and 3.7% (1/27) in extensive stage, suggesting that this point mutation might be an early biologic marker for the disease. The intron 16 mutation was not related to other clinical features including gender, smoking history, or prognosis. As there was only one case of advanced SCLC presenting the point mutation, further study including more patients in extensive stage would be useful to determine the true function and clinical value of c-kit mutation in SCLC lung cancer.

In addition to the analysis of c-kit mutational status, we performed immunohistochemical analyses from the SCLC tumor blocks available. Our study showed that the CD117 expression with moderate to strong staining was demonstrated in a significant number of tumor specimens (56.1%), similar to that reported previously.[7,16] The percentage of cases positive for KIT expression was not significantly different between limited-stage and extensive-stage disease (57.5% vs. 51.9%, $P > 0.05$). One report, however, found a lower incidence of KIT positivity (37%) in SCLC tumor specimens.[17] The discrepancy in data is probably attributable to different samples sources and the scores used in evaluating KIT positivity. In our study, tissue samples were derived from surgical resection and serial biopsies obtained from bronchoscopy and/or mediastinoscopy, which allowed us to compare KIT expression between different tumor origins. Surprisingly, a significantly higher positivity of KIT expression was seen in biopsies (65%) as compared to surgical specimens (56.5%, $P < 0.01$), implying that caution should be taken in interpreting KIT expression when tumor samples were excised by different methods. The OS analysis in our series of SCLC demonstrated that KIT expression was not predictive. Moreover, the prognostic impact of KIT expression on progression-free survival (PFS) was investigated in our work for the first time, and as found for OS, there was no correlation between KIT expression and PFS. Our findings are in agreement with previous studies showing that KIT expression was not related to clinical outcomes.[4,7] Further analysis revealed that KIT expression was not associated with any common clinical parameters, including sex, age, performance status, and disease stage, which represent established prognostic factors for survival in SCLC.[4] In our work, disease stage and treatment status had a significant influence on OS.

Conclusion

In summary, our findings demonstrated significant ethnic differences with respect to frequency and type of c-kit mutations in SCLC. Our work showed that SCLC patients of Chinese ethnicity did not carry any mutations in the c-kit gene in exon 9 or exon 11. Similarly, there were no activating mutations in exon 13 or exon 17. However, a point mutation within intron 16 was observed in the tumor specimens analyzed in this study and to our best knowledge this mutation has not been reported previously. Although the mutation did not predict the expression of KIT, it represents a variation among tumor specimens that should be further investigated in the future. On the basis of these observations, we conclude that the incidence of c-kit mutations in tumor samples in a Chinese cohort was rare and KIT overexpression in our SCLC specimens was not associated with an activating mutation.

Therefore, other mechanisms are probably responsible for KIT overexpression in SCLC.

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Disclosure

The authors declare no conflict of interest.

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